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特　　集

Isolation and identification of human skin fibroblast cell growth factor (SFGF) from the scallop shells

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We have previously shown that scallop shells contain bioactive components. In particular, the scallop shell extract promoted the growth of human skin fibroblast cells and increased in the synthesis of collagen in the fibroblast cells. However, the bioactive substance in the scallop shell remains has been unidentified. In this study, we performed the isolation and identification of human skin fibroblast cell growth factor (SFGF) from scallop shell. The SFGF was purified by a gel filtration column (G-2000SW) and subsequently reverse-phase column chromatographies (C4 and C18). The isolated SFGF was presumed to be a glycopeptide with a molecular weight below 3000 Da. This is the first report about the isolation of a bioactive substance from scallop shell.

Key words: efficient utilization, scallop shells, skin fibroblast cells

1　INTRODUCTION

Shells are composed of two kinds of $CaCO_3$, polymorphs of prismatic layer and nacreous layer. The nacreous layer (pearl) has long been used as Chinese medicine for keeping skin moist, counteracting poison, and making the spirit stable from ancient times. The nacreous layer is also used as cosmetics in Japan.[1-3]

Scallop shells of approximately 300,000 tons per year are generated as industrial waste in Hokkaido (Japan) and recycling of the scallop shells is strongly desired. We previously reported that the scallop shell contains organic components (scallop shell extract) which are useful for protecting the skin. In particular, the scallop shell extract showed growth-promoting activity for human skin fibroblast cells *in vitro*.[4,5] Skin fibroblast cells secrete matrix proteins such as collagen and elastin, which are necessary for the maintenance of tensile strength of skin. Therefore, the SFGF may be useful as a material increasing the amount of collagen and elastin in skin. In this study, we report the isolation and identification of SFGF from scallop shell.

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2　MATERIALS AND METHODS

2．1　Materials:

Scallops were collected from Hunka Bay, Hokkaido. Dulbecco's modified Eagle's medium (DMEM) and Fetal calf serum (FCS) were purchased from Gibco (New York, USA). The 3-(4,5-dimethyl) ethiazole (MTT) was from Wako Pure Chemical Co. Ltd. (Osaka, Japan). ABEE (*p*-aminobenzoic acid ethyl ester) labeling kit was purchased from J-Oil Mills (Tokyo, Japan). Centrifugal filter unit (Ultracel YM3) was from Millipore (Massachusetts, U.S.A). Human skin fibroblast cells (TIG-103) were purchased from the Japanese Collection of research Bioresources (Osaka, Japan).

2．2 Extraction of the scallop shell components:

Scallop shells (approximately 200 g) were brushed clean in order to remove any adhered material, and then crushed to a powder. The powdered shells were completely decalcified using 5 % acetic acid as described previously.[4] The decalcified solution was dialyzed against deionized water in order to remove the acetic acid and lyophilized. The sample was extracted again in deionized water and was employed as the scallop shell extract.

2．3 Cell culture:

The cells were maintained in DMEM supplemented with 10 % FCS in atmosphere of 95 % air and 5 % CO_2. Growth assay of skin fibroblast cells was performed according to the modification of the previously described method.[4] Skin fibroblast cells were seeded at a density of 4 x 10^3 cells in a volume of 100 μL per well in a 96 well plate. After 24 h, the scallop shell extract or each fraction after column chromatography was added to culture medium at various concentrations. The cells in growth phase were treated for 96 h with the scallop shell extract or each fraction after column chromatography, and then the cell numbers were quantified by MTT assay.[6] In brief, MTT (0.5 mg/mL in phosphate-buffered saline [PBS]) was added to each well and incubated for 4 h at 37 degree. The medium were then carefully aspirated, and 150 μL of 20 % sodium dodecyl sulfate was added to solubilize the colored product. After 24 h, absorbance at 570 nm was measured.

2．4 Amino acid composition analysis:

The purified fibroblast cells growth factor was hydrolyzed at 110 degree under vacuum with 6 N HCl for 24 h.[7] The hydrolysate was analyzed on a JLC-500V.

2．5 Monosaccharide composition analysis:

The monosaccharide composition of the purified SFGF was analyzed using an ABEE labeling kit. The subsequent processes of acid hydrolysis, N acetylation, and conversion with ABEE were carried out according to the method of Yasuno *et al*.[8] The resultant ABEE-converted monosaccharide(s) in the aqueous layer was analyzed by reverse-phase HPLC using a Honenpak C18 column according to the manufacturer's instructions. For quantification of monosaccharides, a set of monosaccharides including glucose, galactose, mannose, arabinose, ribose, fucose, xylose, rhamnose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylmannosamine was used as standards.[9]

3 RESULTS AND DISCUSSION

3．1 Growth-promoting activity for human skin fibroblast cells of the scallop shell extract

First, we investigated the effect of the scallop shell extract on the growth rate of human skin fibroblast cells. After the scallop shell extract was supplied to culture medium for skin fibroblast cells, the number of viable cells was investigated by MTT assay. We previously reported that the treatment of human skin fibroblast cells for 24 h with scallop shell extract did not promote the cell growth rate.[4] However, the scallop shell extract could significantly enhance the growth rate of fibroblast cells in a dose-dependent manner when the fibroblast cells were treated with the scallop shell extract for 96 h under the conditions described in *Materials and Methods*. At a concentration of 535 μg/mL (dry w/v), the scallop shell extract showed the number of viable cells increased to approximately 190% compared to that of the control (Fig. 1). These results suggest that the substance in the scallop shell extract may act as growth factor for skin fibroblast cells.

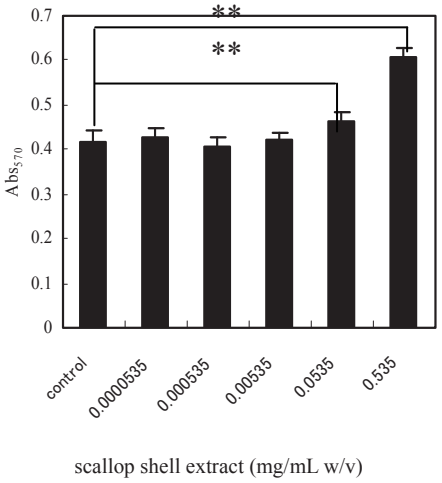

Fig. 1 Effect of the scallop shell extract on the growth of human skin fibroblast cells in vitro

Human skin fibroblast cells (TIG-103) were treated with the scallop shell extract for 96 h. Control cultured cells were treated with an equal volume of the vehicle. The number of living cells was determined by MTT assay. Human skin fibroblast cells were treated (1) in the absence or (2) the presence of 0.0535 μg/ml, (3) 0.535 μg/ml, (4) 5.35 μg/ml, (5) 53.5 μg/ml, and (6) 535 μg/ml of the scallop shell extract. Data were combined from four wells of a 96-well plate and the bars show the mean ± SE. Statistical significance was determined by Student's *t*-test. ** shows p <0.01 relative to vehicle alone.

３．２ Purification of the SFGF

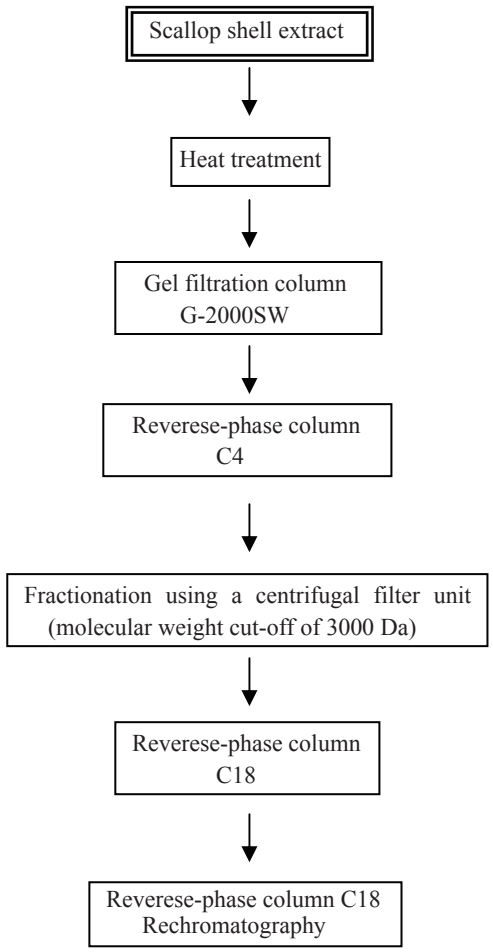

Fig. 2 Preparation scheme of SFGF.

Preparation scheme of SFGF are shown in Fig. 2. The scallop shell extract was heat-treated at 100 degree for 15 min and centrifuged to remove the denatured proteins. The growth-promoting activity was retained in the supernatant, suggesting that SFGF is a heat-stable material. Next the supernatant was separated by a gel filtration column chromatography (G-2000SW) and then subsequently a reverese-phase column chromatography (Shodex 5C4). The fraction with high viscosity was recovered and fractionated using a centrifugal filter unit (Ultracel YM3) with a molecular weight cut-off of 3,000 Da. Finally the fraction with a molecular weight below 3,000 Da was separated by a reverse-phase column chromatography (Megapak SIL C18T-10) and the fraction showing growth-promoting activity was recovered as a single peak (Fig. 3). After the further purification by rechromatography , the pooled fraction was used as purified SFGF in the following analysis.

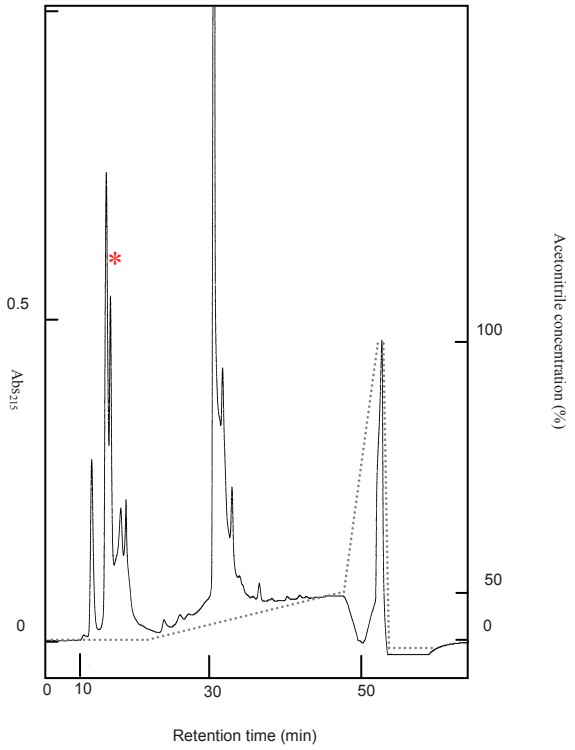

Fig. 3 The final purification step of SFGF

The fraction with a molecular weight below 3,000 Da was separated by C18 reverese-phase column. The column was equilibrated with 0.1% trifluoroacetic acid and the absorbed materials were eluted in a linear concentration gradient of acetonitrile from 0% to 50%. The peak showing growth-promoting activity was represented by an asterisk.

３．３ Amino acid composition and monosaccharide analysis of SFGF

The amino acid composition analysis showed that SFGF was rich in Gly, Glu, Ser, and Asp residues. The amino acid composition is similar to those of MSP-2 and MSP-SC, which we previously identified in the scallop shell,[7,10] suggesting that SFGF is derived from scallop shell. The monosaccharide composition analysis showed that this protein has sugars of glucose, mannose, *N*-acetylglucosamine, and uronic acids (Table. 1). The structure of SFGF-bound oligosaccharide may be new component, because few ***N***-linked oligosaccharides containing several glucoses are known.

Table. 1 The amino acid composition analysis and monosaccharide composition analysis of SFGF were performed as described in *Materials and methods*.

Amino acid	mol ratio
Gly	4.1
Glu	3.8
Ser	2.8
Asp	2.4
Thr	1.2
Ala	0.85

monosaccharide	mol ratio
Glc	5.4
Man	4.2
GlcNAc	2.0
GlcA or GalA	1.7
Gal	1.3
Fuc	0.86

4 CONCLUSION

In this study, we isolated SFGF from scallop shell. The purified SFGF was presumed to be a glycopeptide with a molecular weight below 3,000 Da. The amino acid composition was rich in Asp, Ser, Glu, and Gly residues and contained sugars of glucose, mannose, *N*-acetyl glucosamine, and so on. Now we are trying to determine the amino acid sequence and the oligosaccharide structure of SFGF.

It will be necessary to investigate whether the purified SFGF has abilities increasing of collagen content in the skin and promoting the wound healing of skin *in vivo*. In addition, we will clarify the action mechanism of SFGF for fibroblast cells for the utilization as a cosmetic material in future.

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ホタテガイ貝殻に含まれる皮膚真皮線維芽細胞増殖因子の単離･同定

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概要

ホタテガイ貝殻は毎年 30 万トンにも及ぶ量が産業廃棄物として処分されており、その有効利用を目指し様々な試みが行われている。ホタテガイ貝殻は約 98%が炭酸カルシウム、残り約 2%は蛋白質や糖等の有機成分（貝殻有機成分）で構成されている。我々は貝殻に含まれる有機成分に着目し、皮膚に関する生理活性作用の探索を行ってきた。特に貝殻有機成分が培養ヒト皮膚真皮線維芽細胞の増殖を促進し、コラーゲン合成を増加することを明らかにしてきたが、生理活性物質の同定には至っていなかった。今回我々は貝殻有機成分に含まれる皮膚真皮線維芽細胞の増殖因子の単離、同定を試みた。その結果、低分子量の糖ペプチドであることが推定された。

キーワード：有効利用、ホタテガイ貝殻、皮膚真皮線維芽細胞

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